American Fern Journal 79(4): 143–145 (1989)

Recovery of *Botrychium* Gametophytes, Gemmae, and Immature Sporophytes by Centrifugation

NANCY A. MASON and DONALD R. FARRAR
Department of Botany, Iowa State University, Ames, Iowa 50011

The search for underground gametophytes of pteridophytes is often a tedious and time-consuming process. Their detection is generally dependent upon locating young attached emergent sporophytes (Bruce & Beitel, 1979). Thereafter, soil samples must be carefully searched in order to recover the gametophytes (Mesler, 1976). Gametophytes may be easily broken away from sporophytes or broken into pieces, making recovery laborious. The possibility exists that gametophytes from which sporophytes have not yet developed may be missed in the search.

The fact that locating subterranean plants depends upon finding an emergent sporophyte is especially problematic. We found this to be so while investigating the ecology, reproduction and distribution of *Botrychium campestre* Wagner & Farrar.

In species of *Botrychium*, underground gametophytes and young sporophytes deriving nutrition from a mycorrhizal fungus association may grow for some years without developing emergent leaves, and even after achieving maturity, may not produce aboveground leaves every year. In censusing populations, many individuals may be missed entirely if only plants with emergent leaves are tallied. Excavation is required to locate non-emergent sporophytes.

Population census of *Botrychium campestre* is complicated by this species' ability to reproduce vegetatively underground by means of gemmae (Farrar & Johnson-Groh, 1986). As many as 600 gemmae may be found in association with a single emergent sporophyte leaf, as well as gametophytes and many subterranean sporophytes in various stages of development.

We are currently investigating the distribution of gemmae and non-emergent sporophytes of this and related species. In order to obtain a quantitative estimate of the population size, including isolated gametophytes and non-emergent sporophytes, we have utilized a centrifugation method to recover underground plants and propagules. The method was originally developed to isolate nematodes from soil samples (Jenkins, 1964). We present this method as an effective and efficient way to study the natural occurrence of gemmae, gametophytes, and non-emergent sporophytes.

MATERIALS AND METHODS

The centrifugation method uses soil sieves (available from most biological supply companies) to separate large plant material from soil and to further separate the soil and small plant parts into coarse and fine fractions. We use U.S. Standard soil sieves nos. 10, 35 and 60, which are 203 mm (8 inch) in diameter and have sieve openings of 2 mm, 0.5 mm, and 0.25 mm, respectively. A 76 mm (3 inch) diameter no. 400 sieve with 0.038 mm openings is convenient for collecting and washing sucrose suspended plant matter.

In our investigation of the distribution of *Botrychium campestre*, we collected 48 soil samples from a site where emergent sporophytes were present. Samples were taken with a bulb planter (5 cm diameter), assuring a uniform sample volume of approximately 100 cubic cm.

In the recovery procedure, soil samples are carefully teased apart in a bucket of water, and then passed through a no. 10 sieve into a second bucket. The material retained by the sieve is set aside for later examination. The material which has passed into the second bucket contains gemmae, gametophytes and immature sporophytes in a slurry of several liters of water and 40 to 60 ml of soil. This slurry is then washed through no. 35 (openings 0.5 mm) and no. 60 (openings 0.25 mm) sieves to concentrate the remaining soil and plant material into coarse and fine fractions. Subsequent steps take advantage of the fact that most live plant material, including *Botrychium*, sinks in water, but floats in a 30% solution of sucrose.

The retained soil and plant material is next partitioned into 50 ml centrifuge tubes such that each tube contains about 5 ml of soil in about 40 ml of water. The tubes are stirred and then spun at 2000 rpm for 3 minutes, after which the water and floating debris is decanted. (This supernatant should not contain *Botrychium* fragments but may be saved for examination under the dissecting microscope.)

The material remaining in the tubes is resuspended in a 30% solution of sucrose, then again centrifuged and decanted as before. The decanted liquid, containing *Botrychium* and other live plant material, is washed on a no. 400 sieve and then backwashed into a petri dish for examination under the dissecting microscope.

As a check on the efficiency of recovery, we "seed" each soil sample with stained gemmae that we collect elsewhere. Ten of these are placed in each soil sample we process. We stain gemmae with any of the following 4 stains: propyl carmine, acid fuchsin, aceto-orcein or neutral red. Propyl carmine has proved most effective in staining gemmae a bright, unmistakable red.

As much particulate matter is washed down the drain in the initial reduction of the soil mass, it is necessary to work at a sink adapted for soil collection.

Results

With practice, processing a soil sample takes about 75 minutes. Sieving reduces the initial soil mass by about 90%. After centrifugation, usually less than a gram of material remains to be examined. Finding propagules is greatly facilitated by this reduction in the amount of material. Gemmae, gametophytes and sporophytes in various stages of development are easily identified.

We found 2 gametophytes, 568 gemmae, and 17 non-emergent sporophytes in the 48 samples we processed. We also recovered 80% of the stained gemmae used to "seed" each sample. Thus we feel confident that utilization of this technique results in the successful retrieval of isolated propagules, even when their density is low, and that this method can be profitably used in other studies of reproductive biology of pteridophytes with underground propagules.

Literature Cited

Bruce, J. G. and J. M. Beitel. 1979. A community of *Lycopodium* gametophytes in Michigan. Amer. Fern J. 69:33–41.

Farrar, D. R. and C. L. Johnson-Groh. 1986. Distribution, systematics, and ecology of *Botrychium campestre*, the prairie moonwort. Missouriensis 7:51–58.

Jenkins, W. R. 1964. A rapid centrifugal-floatation technique for separating nematodes from soil. Pl. Dis. Reporter 48:692.

Mesler, M. R. 1976. Gametophytes and young sporophytes of *Ophioglossum crotalophoroides* Walt. Amer. J. Bot. 63:443–448.

Appendix: Detail of Recovery Procedure for *Botrychium* Gemmae

Place the soil sample in a bucket 1/4–1/2 full of water. Gently separate the root mass, freeing the soil from the roots. "Seed" the sample with 10 stained gemmae.

Place a wire rack sieve holder in a second bucket and rest the no. 10 sieve on it. Pour the bucket of soil and water through the no. 10 sieve into the second bucket, rinsing out the first bucket with water from the tap (through a tube attached to the spigot). Rinse the root mass on the sieve, catching the rinse water and soil particles in the second bucket. Set aside the rinsed material for later examination. Plants and propagules may be present.

Stack the no. 35 and no. 60 sieves, the no. 35 on top. Pour the contents of the second bucket through them. Rinse over the soil sink or over a bucket. Although the rinse water will not be saved, rinsing over a bucket with the sieves atop a wire rack allows for maximum air flow through the sieves and reduces the chance of water backing up through the screens. Overflow can be a problem with fine, silty soils; the overflow can be caught by the bucket and recycled through the screens. While rinsing, use fingers to gently reduce the material.

When the soil will no longer reduce (rinse water does not appear muddy), rinse it down into one edge of the screen. When saving the contents of the coarse screen (no. 35), backwash the material first onto a finer screen (no. 100, 0.15 mm openings) for easier handling. Gemmae will pass through the no. 35 sieve, so material must be transferred carefully to the no. 100 screen.

Using a spatula, transfer screen contents into centrifuge tubes. The remainder of particles on the screen can be washed with a water bottle into a small beaker and transferred to the tubes. We use 4 to 8 tubes depending on the amount of material. Tubes should not be filled more than 1/5 full of soil.

Add water to nearly fill the tubes. Stir the tube contents. Balance tubes before centrifuging. Spin the tubes for 3 minutes at 2000 rpm. Decant the liquid through a no. 400 sieve, taking care not to pour off sediment in the bottom of the tube. Backwash the solids retained by the sieve into a petri dish. (This material should not contain live plants, but may contain still identifiable dead material.)

Resuspend the soil in a 30% sucrose solution. (After pouring off the supernatant, sediment will adhere to the sides of the centrifuge tubes. This can be washed down with 30% sucrose in a wash bottle). Stir and balance the tubes. Spin for 3 minutes at 2000 rpm. Decant the liquid through the no. 400 sieve and use a water bottle to carefully wash the sides of the tilted tube. This captures any gemmae or material which may get stranded on the wall of the tube. Backwash sieve contents into a petri dish. (The sucrose suspension and centrifugation may be repeated to increase probability of propagule recovery. We suspended each sample in sucrose twice, though most of the material is decanted in the first sucrose suspension.) Examine retained material under a dissecting scope.